SEPARATION AND IDENTIFICATION OF LIME CUTIN MONOMERS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND MASS SPECTROMETRY

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Key Word Index—*Citrus aurantifolia*; citrus cuticle; cutin; analysis; HPLC; GC-MS; CIMS; CPMAS NMR.

Abstract—A recently developed HPLC technique has been used to identify 10 major monomers from potassium hydroxide hydrolysis and transesterification of lime cutin, revealing 16-hydroxy-10-oxo-hexadecanoic and 10,16-dihydroxyhexadecanoic acids as the major constituents. Solid-state ^{13}C NMR spectroscopy has also been used to examine the unreacted residues following hydrolytic treatment. For transesterification with methanolic boron trifluoride, an alternative protocol using MS has been developed. Using HPTLC to separate epoxy and hydroxy fatty esters, and making a series of trimethylsilyl ether derivatives, the monomeric products have been subjected to analysis by GC-CI-mass spectrometry. Although GC cannot discriminate between positional isomers of oxo and dihydroxy fatty acids, the parent ions and fragmentation patterns obtained with CI-mass spectrometry allow definitive identification of each isomer and reveal five new constituents of the cutin biopolymer. Both the methodology and the monomeric structures are compared with prior reports for citrus fruit cuticle; implications for the molecular architecture and biosynthesis of the lime cutin polymer are also discussed.

INTRODUCTION

Because cutin and cuticular wax play a key mediating role between higher plants and the environment, their chemical composition and structure have been the subject of numerous investigations [1–4]. Partial depolymerization of the cutin polyester has been accomplished using various chemical and enzymatic methods, including hydrogenolysis with $LiAlH_4$, hydrolysis with alcoholic KOH or HCl and breakdown with cutinases [1, 2, 5]. Structural identification of the products of these reactions is often laborious, and it may fail to discriminate among epoxide, oxo and alcohol groups in the original cutin polymer. Nonetheless, a comprehensive database of monomeric constituents is essential for investigations of cutin molecular structure that are based on enzymatically generated oligomeric fragments.

In order to separate all major products from base hydrolysis of lime cutin without the need for derivatization, a newly developed high-performance liquid chromatography (HPLC) method with an evaporative light-scattering detection (ELSD) [6, 7] has been evaluated herein with the cutin polymer from lime fruits. The corresponding unreacted material has been examined using ^{13}C NMR spectroscopy. Alternatively, a transesterification treatment as reported for the study of potato wound periderm [1, 3] has been used to produce methyl esters from carboxylic groups and methoxyhydrins from epoxide groups, respectively, leaving the oxo groups within the polymer structure unaltered. In the latter study, gas-liquid chromatography in combination with chemical ionization mass spectrometry (GC-CIMS) has been used to confirm the identifications made by HPLC, to distinguish positional isomers spectroscopically and to identify several new lime cutin constituents. In the present study, both of these routes to cuticular monomers of lime fruits have been compared with respect to their chemical consequences, ease of use and ability to identify structurally similar monomeric constituents.

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RESULTS

HPLC analysis of monomers from alkaline hydrolysis

The yield of fatty acids from an 8 hr alkaline hydrolysis of lime cutin (60–65%) is lower than that reported previously for other fruit cutins [7], though it increases to the expected 80% if the reaction time is extended to 22 hr.

This unusual resistance to hydrolytic breakdown, which has also been observed for lime cutin upon treatment with porcine pancreatic lipase, may be attributed to cross-links within the polymeric structure [3] that limit its enzymatic accessibility.

By comparing HPLC R_t values for the hydrolysis products of lime and apple cutin, as well as for reference compounds [6, 7], it is possible to identify with reasonable certainty the 10 major monomers listed in Table 1. The predominance of 16-hydroxy-10-oxo-hexadecanoic acid (G) agrees with prior studies of citrus fruit cutin, in which large proportions of ω-hydroxyoxo C_{16} acids were found among the hydrogenolysis and alkaline hydrolysis products of grapefruit, lime, lemon, orange, clementine and mandarin [8, 9]. The other major constituent of lime cutin, 10,16-dihydroxyhexadecanoic acid (I), was reported as an important monomer in citrus, tomato and green pepper [7]. In addition, the lime fruit cutin includes significant amounts of C_{16} and C_{18} carboxylic acids (A), 10-hydroxyhexadecanoic acid (B) and 16-hydroxyhexadecanoic acid (E), which have been omitted from most discussions of cuticular composition. Three minor unidentified monomers are also observed by HPLC (data not shown).

Positional isomers such as the pairs (F, G) and (H, I) are distinguished readily in lime cutin using the HPLC protocol. The mid-chain-oxygenated substituent in these fatty acid derivatives is roughly 10 times more likely to be found at the 10-position than the 9-position, a trend in line with the qualitative results obtained using more traditional GC-MS methods [7; see below].

Solid-state NMR analysis of unreacted residues from alkaline hydrolysis

A complementary view of lime cutin hydrolysis is obtained from examination of the unreacted solids from the treatment. Figure 1 compares high-resolution ^{13}C NMR data for intact cutin and the unreacted residues after 8 and 22 hr, respectively. In contrast to the broad cutin lines attributed previously to a dispersion of chemical environments within an irregular polymer, the narrower signals observed in spectra of the lime cutin residue are consistent with removal of the monomers described above to yield a more homogeneous material [3]. In the bulk methylene region, for instance, the carbons that resonate at 25 and 41 ppm are absent from ^{13}C NMR spectra of the residues. Although the intact cutin contains significant numbers of mobile methylene and carbonyl carbons that give NMR signals under low-power decoupling conditions [3], its residues consist almost exclusively of rigid, solid-like functional groupings (data not shown).

As the hydrolysis proceeds, the CPMAS ^{13}C NMR spectra reveal that particular carbon types are removed or retained. The carbonyl signals from secondary esters (173 ppm) are progressively and strikingly diminished in size, whereas the carbonyls of primary esters (168 ppm) are retained in the lime residue. Concomitantly, the CHO-signal intensity (72 ppm) increases with respect to the $(CH_2)_n$ resonances (29 ppm). These two observations suggest that KOH preferentially hydrolyses esters of secondary alcohols, thus converting CHOC=O to CHOH groups within the solid residue. As these CHOH groups are produced, the $(CH_2)_n$ signals are diminished by the removal of long-chain fragments, which ultimately appear as soluble monomers e.g. F–I. The aromatic or unsaturated resonances in the ^{13}C NMR spectrum (115 and 129 ppm) are also reduced in intensity, though we identified only minor monomers that contain these functional groups.

HPLC analysis of monomers from transesterification

A mild treatment of lime cutin with boron trifluoride–methanol gives a 50% yield of monomeric methyl esters, comparable to the highest percentages reported with suberized potato tissue as a substrate [1], but noticeably lower than yields from our alkaline hy-

Table 1. HPLC-ELSD analysis of lime cutin monomers*

Monomeric constituent†	Acids		Methyl esters	
	R_t (min)	Area (%)	R_t (min)	Area (%)
A: hexadecanoic, octadecanoic	2.4	3.7	2.4	3.9
B: 10-hydroxyhexadecanoic‡	9.5	0.95	6.3	7.8
C: 18-hydroxyoctadeca-9,12-dienoic	13.8	0.8	11.4	0.5
D: 18-hydroxyoctadeca-9-enoic	14.4	1.7	12.5	4.4
E: 16-hydroxyhexadecanoic	15.5	2.8	13.2	4.3
F: 16-hydroxy-9-oxo-hexadecanoic	21.7	4.8	19.2	2.0
G: 16-hydroxy-10-oxo-hexadecanoic	22.7	51.0	20.2	30.2
H: 9,16-dihydroxyhexadecanoic	26.6	2.9	25.1	3.2
I: 10,16-dihydroxyhexadecanoic	28.1	27.9	26.8	29.4

* Performed using a Chrompack Si60 column with a binary (hexane–isopropanol) gradient, as described in the Experimental.

† Three additional minor peaks were eluted after monomer A and before monomer B.

‡ This tentative identification is derived from R_t values for free monohydroxy fatty acids [6].

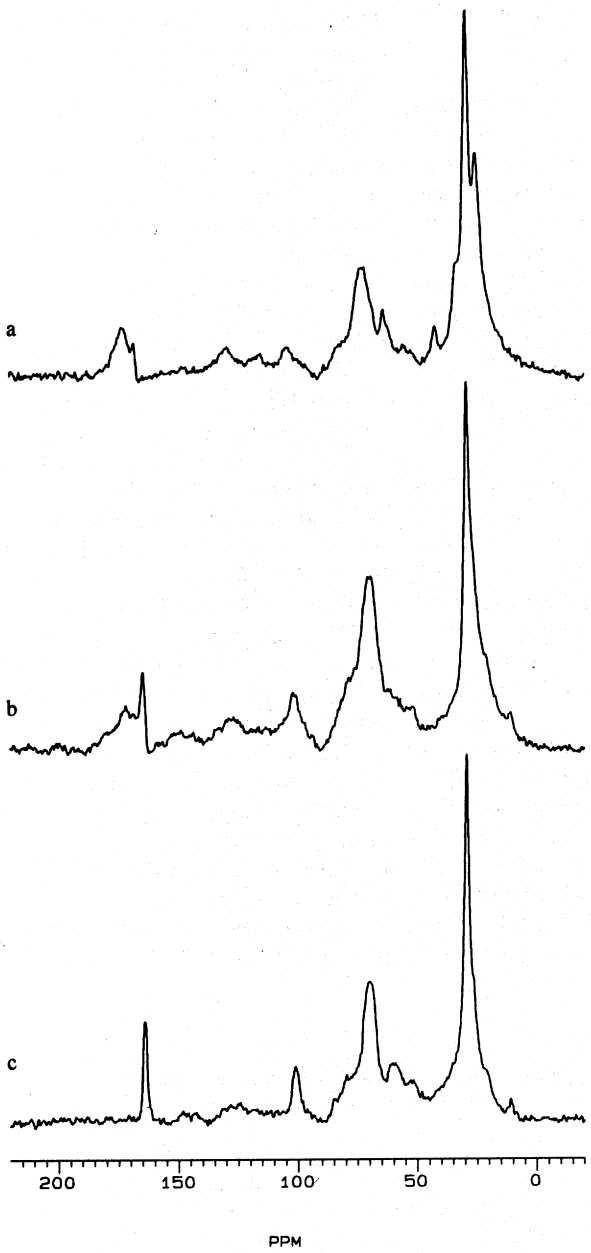


Fig. 1. ^{13}C CPMAS NMR (50.33 MHz) spectra of lime cutin obtained (a) before treatment, (b) after 8 hr treatment with methanolic KOH, and (c) after 22 hr treatment with methanolic KOH. A total of 6000 transients were averaged for each spectrum, using a spectral width of 20 kHz defined by 2 K data points. After zero-filling to 4 K points, each spectrum was processed with a digital line broadening of 20 Hz and plotted with the largest peak set to full scale. The chemical-shift assignments are as follows: $(CH_2)_n$, 29 ppm; $\underline{C}H_2CH_2OCOR$, 42 ppm; $CH_2\underline{C}H_2OCOR$, 63 ppm; $\underline{C}HOCOR$ and CHOH, 72 ppm; aromatic/unsaturated, 115 and 129 ppm; carbonyl, 168 and 172 ppm [3].

drolysis of the cutin. When compared to alkaline hydrolysis, this latter procedure produces more volatile methyl esters of the constituent fatty acids and also converts epoxides in the polymer structure to the corresponding methoxyhydrins. The R_t values for the methyl esters are *ca* 2 min shorter than for the corresponding free acids [6], facilitating their identification from HPLC-ELSD data on the parent carboxylic acids (Table 1). Quantitatively, the relative concentration of products from the transesterification treatment (as determined by HPLC) confirms the importance of monomers G and I, as well as the predominance of isomers with midchain substitution at the 10-position. When compared with the alkaline hydrolysis products, however, the transesterification procedure results in relatively fewer oxo derivatives and more dihydroxy C_{16} monomers.

Separation and MS analysis of monomers from transesterification

Table 2 shows the results of analysis of the lime cutin monomers derived from treatment with boron trifluoride–methanol using either high-performance thin-layer chromatography (HPTLC) or gas chromatography of derivatives. The expected differences in R_t and R_f values are observed between monohydroxy fatty acids (B, D, E, J, K) and dihydroxy fatty acids (H, I, M, N), and between dihydroxy fatty acids with different chain lengths or substitution patterns (H, I; M, N). Unique chromatographic behaviour is observed for oxo compounds (F, G) and for methoxyhydrins (L). Nonetheless, these methods fail to resolve positional isomers such as (F, G), (H, I) and (M, N).

The identities of the lime cutin monomers listed in Table 2 are derived from GC analysis in combination with chemical-ionization-mass spectrometry (CIMS) of their trimethylsilyl (TMSi) derivatives (Table 3). These constituents include the monomers identified tentatively from HPLC-ELSD (above) and five additional molecular species for which authentic standards are unavailable. Using methane as the reagent gas for CIMS, initial identifications of these compounds come from observations of the molecular ion ($[M + H]^+$), adduct ions ($[M + 29]^+$ and $[M + 41]^+$), and elimination ions ($[M - 15]^+$ and $[M - 31]^+$). In some instances $[M - H]^+$ and $[M - H - MeOH]^+$ ions also appear in the mass spectrum [10–12]. Identification of the epoxy fatty acids and discrimination between the positional isomers rely on diagnostic α-cleavages observed for silylated (TMSi) derivatives, which may be obscured by more complex fragmentation in traditional EIMS spectra, but are more apparent with methane CIMS [12–14]. No reference standards are required.

For instance, transesterification products based on 16-hydroxy-9-oxo-hexadecanoic acid (F) and its positional isomer 16-hydroxy-10-oxo-hexadecanoic acid (G) elute together from the GC column at 16.1 min. CIMS of their TMSi ethers shows prominent peaks corresponding to $[M + H]^+$ at m/z 373, $[M + 29]^+$ at m/z 401, $[M + 41]^+$ at m/z 413, $[M - 15]^+$ at m/z 357 and $[M - 31]^+$ at m/z 341, respectively (Table 3). The presence of a hydroxyl function is evident from strong peaks at m/z 283 $[MH - TMSOH]^+$ and m/z 251 $[M - TMSOH - MeOH]^+$. The two oxo isomers F and G are detected and distinguished by the appearance of ions at m/z 185 and 199, which result from the cleavage of a carbon–carbon bond adjacent to their respective carbonyl groups. The relative intensities of these last two ions confirm that G is more abundant than its positional isomer F, as indicated above by HPLC analysis of the KOH hydrolysis products.

The second major constituent of the lime cutin consists of 9,16- and 10,16-dihydroxyhexadecanoic acids (H and I), eluted from the GC column at 13.5 min. CIMS gives peaks corresponding to $[M - H]^+$ at m/z 445, $[M + 29]^+$ at m/z 475, $[M + 41]^+$ at m/z 487, $[M - 15]^+$ at m/z 431 and $[M - 31]^+$ at m/z 415. As expected, these isomeric mixtures show peaks from cleavages of the primary TMSi ethers, m/z 357 from cleavage a and m/z 371 from cleavage b, respectively. The positions of the secondary hydroxyl functionalities are revealed by cleavage c, which yields ions at m/z 259 and 289 from H, as compared with m/z 273 and 275 from I. The intensities of these

Table 2. Chromatographic separation of lime cutin methyl esters*

Monomeric constituent	GC	HPTLC
	R_t (min)†	R_f
B: 10-hydroxyhexadecanoic	11.6	0.47
D: 18-hydroxyoctadeca-9-enoic	14.8	0.49
E: 16-hydroxyhexadecanoic	14.7	0.47
F: 16-hydroxy-9-oxo-hexadecanoic	16.1	0.38
G: 16-hydroxy-10-oxo-hexadecanoic	16.1	0.38
H: 9,16-dihydroxyhexadecanoic	13.5	0.10
I: 10,16-dihydroxyhexadecanoic	13.5	0.10
J: 16-hydroxyhexadeca-*n*-enoic	14.7	0.47
K: 18-hydroxyoctadecanoic	14.8	0.49
L: 9,18-dihydroxy-10-methoxy-octadeca-*n*-enoic‡	16.2	0.14
M: 10,11-dihydroxyoctadioic	14.3	0.09
N: 9,10-dihydroxyoctadioic	14.3	0.09

* Gas chromatography carried out using a silica capillary column; high-performance thin-layer chromatography performed using silica gel 60 plates developed with CH_2Cl_2–hexane–MeOH. See Experimental for details.

† Conducted for TMSi ether derivatives of the methyl esters produced by transesterification with BF_3–MeOH.

‡ Corresponds to constituent C produced by cutin hydrolysis with alcoholic KOH.

fragment peaks indicate that I is more abundant than its positional isomer H, again in accord with HPLC results for the parent acids.

Compound E and its monounsaturated analogue J are eluted at 14.7 min in the gas chromatogram. It is possible to identify E as 16-hydroxyhexadecanoic acid using CIMS peaks at m/z 359 $[M + H]^+$, m/z 387 $[M + 29]^+$, m/z 399 $[M + 41]^+$, m/z 343 $[M - 15]^+$ and m/z 327 $[M - 31]^+$. The diagnostic TMSi ether cleavage yields peaks at m/z 269 (cleavage a) and m/z 237 (cleavage a-MeOH), and cleavage α to the ether produces a smaller peak at m/z 255. The presence of its analogue J, with a mass 2 amu lower, is indicated by a molecular ion peak at m/z 357 and peaks corresponding to the diagnostic cleavages (a–c').

Although the minor components reported for some plant cutins have included a dihydroxy-C_{16} acid (10-oxo-9,16-dihydroxyhexadecanoic) and two monohydroxy-C_{18} acids (10-hydroxyoctadecanoic and 12-hydroxyoctadeca-9-enoic acids) [15, 16], monohydroxy C_{16} acids other than ω-hydroxyhexadecanoic acid have not been cited in the literature or identified in our HPLC analysis of KOH hydrolysis products. Upon transesterification, the small peak eluted in GC at 11.6 min is identified as 10-hydroxyhexadecanoic acid (B). Hydride abstraction [12] produces a molecular ion in the mass spectrum at m/z 357 $[M - H]^+$. The location of the hydroxyl group is deduced from (i) observation of two pairs of α-cleavage ions on either side of the secondary ether group [m/z (273, 85) and (189, 169)]; and (ii) observation of smaller fragments based on $Me\text{-}(CH_2)_5\text{-}CH{=}O$ and $O{\equiv}C\text{-}(CH_2)_8\text{-}COOMe$ (m/z 114, 199, and 169).

Several C_{18} acid constituents of cutin have been reported to contain a double bond at C-9, an epoxide at the 9,10 position or a vicinal diol at the 9,10 position [2]. Our GC-CIMS studies provide supporting evidence for the following C_{18} acids: 18-hydroxyoctadecane-9-enoic acid (D) and its saturated analogue (K), 9,10-epoxy-18-hydroxyoctadecane-12-enoic acid (L), 9,10-dihydroxyoctadecanedioic acid (M), and 10,11-dihydroxyoctadecanedioic acid (N). With the exception of positional isomers, all of these compounds have distinct elution times in GC-MS. As described for the C_{16} acids, the C_{18} analogues are characterized by their molecular ions and diagnostic α-cleavages.

The mass spectrum of 18-hydroxyoctadeca-9-enoic acid (D) and its saturated analogue (K), which co-elute at 14.8 min in the gas chromatogram, shows two molecular-ion peaks at m/z 385 $[M + H]^+$ and m/z 387 $[M + H]^+$, respectively. The terminal TMSi ether cleavages give intense peaks at m/z 297 (cleavage a) and at m/z 265 (cleavage a-MeOH) for the saturated acid (K). The corresponding peaks for the unsaturated acid (D) appear 2 amu lower at m/z 295 and 263, thus confirming its identity. Additional ions produced by α-cleavages b and c' are also observed for both acids D and K. This pair of monohydroxy C_{18} fatty acids is analogous to the C_{16} compounds E and J described above.

The eluate at 13.5 min, which is derived from the HPTLC band with an R_f of 0.10, contains the isomeric dihydroxyhexadecanoic acids H and I as major components and the dihydroxy-dicarboxylic acids 9,10- and 10,11-dihydroxyoctadeca-1,18-dioic acid (M and N) as significant minor components. Thus, although HPTLC fails to separate these structurally similar species, they may all be observed clearly using CIMS. As described above, H and I display a molecular ion at m/z 445 $[M - H]^+$ and prominent related peaks at m/z 475 $[M + 29]^+$, 487 $[M + 41]^+$, 431 $[M - 15]^+$ and 415 $[M - 31]^+$. H and I also show the expected fragments at m/z 357, 371, 289, 259, 275 and 273 amu. For the dihydroxy-dicarboxylic acids M and N, CIMS yields a protonated molecular ion at m/z 519 $[M + H]^+$ and an elimination ion at m/z 487 $[M - 31]^+$. The diagnostic α-cleavage peaks both appear at m/z 259 for the symmetric 9,10-dihydroxy isomer; subsequent loss of the trimethylsilyl group gives a large peak at m/z 185. The corresponding α-cleavage peaks for the 10,11-dihydroxy isomer are m/z 273 and 245; loss of trimethylsilyl groups produces fragments with m/z 199 and 171, respectively. The dihydroxy-octadecanedioic acid has been observed previously as a minor constituent in several fruit cutins [2], but the 10,11 isomer has not been reported.

Finally, the transesterification procedure gives an epoxy compound, identified as 18-hydroxy-9,10-epoxy-octadeca-*n*-enoic acid (L) [2]. (This compound corresponds to the 18-hydroxyoctadeca-9,12-dienoic acid (C) produced by base hydrolysis.) The methyl ester of this epoxy acid elutes from the GC at 16.2 min; it is identified using CIMS by its $[M + H]^+$ ion at m/z 503, its adduct ion at m/z 531 $[M + 29]^+$, and its elimination ions at m/z 487 $[M - 15]^+$ and m/z 471 $[M - 31]^+$. The positions of the two TMSi ethers are deduced by α-cleavage peaks at m/z 399 (cleavage c') and m/z 186 (cleavage c). The mass spectrum supports the formation of just one type of methoxyhydrin (9-OH, 10-OMe) from the cutin polymer during the methanolic boron trifluoride treatment. Although unsaturation at the C-12 position has been reported previously for C_{18} cutin monomers [1, 2], it is also reasonable to place the double bond at the C-11 position, since acid-catalysed ring opening of the epoxide would produce a transition state in which a resonance-stabilized carbocation at the C-10 carbon facilitates nucleophilic attack by methanol.

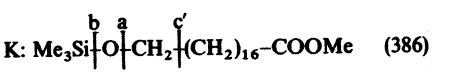
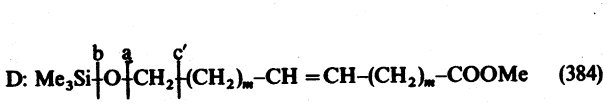
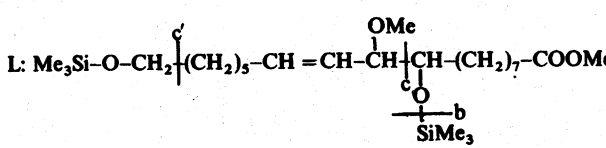
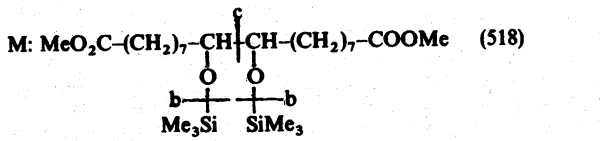
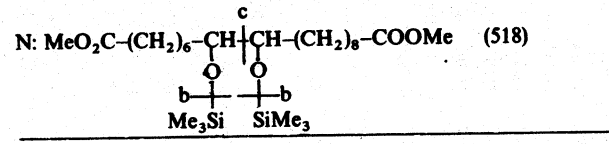
Taken together, the results presented in Table 3 also indicate that primary TMSi ethers are much more susceptible to cleavage than secondary TMSi ethers in CH_4 CIMS experiments. Thus, cleavages a and b produce more abundant ions from primary hydroxy fatty acids (D–K) than from secondary hydroxy fatty acids (B, L–N). This aspect of the fragmentation patterns confirms our CIMS identification of primary and secondary hydroxy acid constituents of the lime cutin.

DISCUSSION

The complementary chemical and analytical strategies employed in this work augment existing structural information about the monomeric constituents, polymeric structure and biosynthetic pathways for lime cutin. For instance, our CIMS studies provide the first evidence for

Table 3. CIMS of TMSi ethers of C_{16} and C_{18} acid monomers from depolymerization of lime cutin with BF_3–MeOH*

Compound (molecular weight)	$[M + H]^+$ or $[M - H]^+$ *m/z* (%)	$[M + 29]^+$ $[M + 41]^+$ $[M - 15]^+$ $[M - 31]^+$ *m/z* (%)	Cleavage a *m/z* (%)	Cleavage b *m/z* (%)	Cleavage c *m/z* (%)	Cleavage c′ *m/z* (%)
E: Me_3Si–O–H_2C–$(CH_2)_{14}$–COOMe (358)	359 (77.2)	387 (43.1) 399 (13.6) 343 (84) 327 (38.6)	269 (72.7) 237 (59)†	285 (6.8),	—	255 (15.9)
B: Me–$(CH_2)_5$–CH(O–$SiMe_3$)–$(CH_2)_8$–COOMe (358)	357 (7.9)	—	—	114 (65.9)†	273 (25), 189 (48.8), 169 (11.3), 85 (31.8) 114 (65.9)‡	—
F: Me_3Si–O–H_2C–$(CH_2)_6$–C(=O)–$(CH_2)_7$–COOMe (372)	373 (65.9)	401 (38.6) 413 (14.7) 357 (70.4) 341 (61.4)	283 (47.7) 251 (68.1)	299 (5.6) 297 (4.5)	185 (18.1)	—
G: Me_3Si–O–H_2C–$(CH_2)_5$–C(=O)–$(CH_2)_8$–COOMe (372)	373 (65.9)	401 (38.6) 413 (14.7) 357 (70.4) 341 (61.4)	283 (47.7) 251 (68.1)	299 (5.6) 297 (4.5)	199 (30.6)	—
J: Me_3Si–O–CH_2–$(CH_2)_m$–CH=CH–$(CH_2)_n$–COOMe (356) $(m + n) = 12$	357 (52.2)		267 (29.5) 235 (43.1)†	283 (10.2)		253 (11.3)
H: Me_3Si–O–H_2C–$(CH_2)_6$–CH(O–$SiMe_3$)–$(CH_2)_7$–COOMe (446)	445 (13.6)	475 (15.9) 487 (6.8) 431 (38.6) 415 (11.3)	357 (70.4)	371 (9)	289 (9) 259 (20.4)	341 (19.4)
I: Me_3Si–O–CH_2–$(CH_2)_5$–CH(O–$SiMe_3$)–$(CH_2)_8$–COOMe (446)	445 (13.6)	475 (15.9) 487 (6.8) 431 (38.6), 415 (11.3)	357 (70.4)	371 (9)	275 (25), 273 (34)	341 (19.4)

K:  (386)		387 (12.5)	415 (9) 427 (4.5), 371 (31.8), 355 (11.3)	297 (34), 265 (9)†	313 (4.5)	—	283 (13.6)
D:  (384)		385 (18.1)		295 (9), 263 (18.1)†	311 (6.8)	—	281 (10.2)
L:  (502)		503 (100)	531 (25.6) 487 (52.7) 471 (37.8)	—	—	186 (10.8)‡	399 (21.6)
M:  (518)		519 (17.8)	487 (7.2)	—	371 (6.8)	259 (10.9) 185 (32.8)‡	—
N:  (518)		519 (17.8)	487 (7.2)	—	371 (6.8)	199 (6.8)‡ 171 (31.5)‡	—

* Performed with a Quadrupole Gas Chromatograph–Mass Spectrometer, operating in chemical ionization mode using methane as the reagent gas.
† Cleavage [a – MeOH].
‡ Cleavage [c – $SiMe_3$].

gradient (hexane–acetic acid–isopropanol, 7:2:991–200:2:778) for elution [6, 7]. A Varex Universal Evaporating Light-Scattering Detector (ELSD) (Rockville, MD) was used for the detection of hydroxy fatty acids, operating at a temp. of 40°C and with N_2 (25 psi) as a nebulizing gas.

Transesterification. Cutin powder (500 mg) was suspended in 66 ml BF_3–MeOH complex (14% w/v of BF_3) and refluxed for 8 hr under N_2. The mixt. was filtered at room temp. and poured into 150 ml of a satd sodium bicarbonate soln. The organic product was extracted with CH_2Cl_2 (3 × 50 ml), dried over anhydrous magnesium sulphate and taken to dryness under red. pres. Based on the mass of methyl ester products, typical yields for the reaction were 48–53%.

Fractionation of the aliphatic esters by HPTLC. The dried extract from the BF_3–MeOH treatment (30 mg) was dissolved in a min. vol. of CH_2Cl_2–MeOH (2:1) and was applied to pre-coated silica gel 60 HPTLC plates (thickness 0.2 mm, 10 × 20 cm; EM Separations, Gibbstown, NJ) with 10 μl micropipettes. The plates were developed with CH_2Cl_2–hexane–MeOH (70:30:2). After visualizing the plates with iodine and UV light, the sepd zones were scraped off and the compounds recovered by elution of the silica gel with a 2:1 mixt. of CH_2Cl_2 and MeOH.

Trimethylsilyl derivatives. Trimethylsilyl ethers were prepd by heating 0.1–0.5 mg of each sepd sample with 50–100 μl of BSA for 30 min at 90°C.

GC-CIMS. The derivatized samples described above were analysed using a Finnigan 3300 Quadrupole Gas Chromatograph-Mass Spectrometer (GC-MS) (San Jose, CA). GC conditions were as follows: 25 m × 0.52 mm fused silica capillary column coated with 1.0 μm OV-1701 (Quadrex Corp; New Haven, Conn.), He flow rate 1.5 ml min^{-1}. The column temp. was set initially at 100° and then increased to 280° at a rate of 20° min^{-1}. Hydroxy and epoxy (methoxyhydrin) fatty acid methyl esters were analysed in a single GC-MS experiment, running in chemical ionization (CI) mode using methane as the reagent gas.

Solid-state NMR spectroscopy. ^{13}C NMR spectra were acquired at 27° on an IBM Instruments (Bruker) WP-200 spectrometer operating at 50.33 MHz, equipped with high-power amplifiers and a magic-angle spinning (MAS) probe from Doty Scientific (Columbia, SC). Lime cutin samples weighing 110–144 mg were ground with a Spex freezer mill (Edison, NJ) and packed into 7-mm cylindrical, double-bearing Zirconia rotors (Doty Scientific). The NMR spectra were obtained with 4.8 kHz MAS, 47 kHz matched ^1H–^{13}C polarization transfer (CP) lasting 1.0 msec, 47 kHz ^1H decoupling during signal acquisition, and a 1 s delay between acquisitions. ^{13}C chemical shifts are quoted with respect to tetramethylsilane, using *p*-di-tert-butylbenzene as a secondary substitution ref.

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